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(54) Method for expressing foreign genes in schizosaccharomyces pombe and the use in therapeutic formulations of the products, DNA constructs and transformant strains of schizosaccharomyces pombe usable in such method and their preparation.

(57) A method is provided for producing heterologous polypeptides in yeast *Schizosaccharomyces pombe*. DNA constructs are provided for use in this method comprising a segment coding for an *S. pombe* promoter. A particular construct is provided comprising the *S. pombe* alcohol dehydrogenase promoter and human alpha-1-antitrypsin gene, which is used to transform *S. pombe* to produce a substantially unglycosylated protein having activity of human alpha-1-antitrypsin.

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METHOD FOR EXPRESSING FOREIGN GENES IN SCHIZOSACCHAROMYCES
POMBE AND THE USE IN THERAPEUTIC FORMULATIONS OF THE
PRODUCTS, DNA CONSTRUCTS AND TRANSFORMANT STRAINS OF
SACCHAROMYCES POMBE USABLE IN SUCH METHOD AND THEIR
PREPARATION

The present invention is directed to methods for expressing heterologous polypeptides in the fission yeast
5 Schizosaccharomyces pombe, to DNA vectors useful therein, and to protein products expressed thereby.

In the field of genetic engineering where the object is normally to obtain high expression of a polypeptide product, usually a mammalian protein, in a micro-
10 organism, the choice of the host organism may be critical. Some work has been done in the eucaryotic organism Saccharomyces cerevisiae (Baker's yeast). However, whether other eucaryotic microorganisms may achieve similar or even higher levels of expression of hetero-
15 logous proteins was not heretofore known. The fission yeast Schizosaccharomyces pombe is only distantly related to S. cerevisiae and the two genera differ in many aspects of biochemistry and physiology. Such differences, for example, may be reflected in possible
20 different levels and/or specificities of proteases in the different species which may degrade heterologous proteins. Also, the yeast genera may differ in enzymes which modify heterologous proteins, which may involve differing specific cleavage sites, sulfhydryl bridges,
25 glycosylation, amino-terminal modifications and the

protein which one may express, such specific modifications may be desirable or undesirable. Furthermore, different yeast genera may contain different proteins which in some cases may be more difficult than others to
5 purify away from the desired heterologous protein. Also, at certain levels the presence of heterologous proteins may have a detrimental effect on cell viability, so it may be desirable to determine which yeast genera may be most tolerant to the particular desired
10 protein. The secretion processes may differ among yeast genera, therefore one may be able to take advantage of such properties, for example, a particular heterologous protein may be secreted at a higher level in one genus versus another, or undesirable proteases and protein
15 modifying enzymes may be secreted at a lower level while the heterologous protein is secreted at a higher level in certain genera. Finally, yeast genera may differ in their growth rates or yields in different media at different temperatures and pressures, thereby affecting
20 time and cost of production. It is therefore desirable to provide a method of producing foreign proteins in different eucaryotic organisms to take advantage of such differences in characteristics. In most cases, such differences in characteristics may not be determined a
25 priori.

It is therefore an aspect of the present invention to provide methods for producing heterologous proteins in the fission yeast Schizosaccharomyces pombe.

It is a further aspect of the present invention to
30 provide DNA vectors which are useful for expressing heterologous proteins in S. pombe.

It is another aspect of the present invention to provide DNA expression vectors comprising S. pombe alcohol dehydrogenase promoter.

5 It is a further aspect of the present invention to provide substantially pure unglycosylated human alpha-1 antitrypsin produced by Schizosaccharomyces pombe.

In the accompanying FIGURES:

FIG. 1 is the cDNA sequence of the gene coding for the predominant form of human alpha-1 antitrypsin.

10 FIG. 2 illustrates a partial sequence of pUC13 including the linker.

FIG. 3 illustrates a method of preparation of vector HAT 4.

15 FIG. 4 illustrates the restriction map of vector C1/1, a vector utilized in preparation of HAT 4.

FIG. 5 illustrates the restriction map of the vector pUC α -1 + FG1, an intermediate vector used in the preparation of HAT 4.

FIG. 6 illustrates the restriction map of HAT 4.

20 FIG. 7 illustrates a method of preparing vector pR103-5, an expression vector for producing alpha-1 antitrypsin in S. pombe.

FIG. 8 illustrates the restriction map of pR103-5.

The present invention is directed to a method for producing a heterologous protein in yeast genus S. pombe comprising the step of growing a culture of the yeast transformed by a DNA vector containing a segment coding for an S. pombe promoter, whereby expression of the protein is under regulation of the promoter. DNA constructs useful for making such vectors are also provided. In particular, a vector is constructed comprising the S. pombe alcohol dehydrogenase promoter and the structural gene for human alpha-1 antitrypsin which is transformed into S. pombe. Growth of the transformant produces a substantially unglycosylated protein having the activity of mammalian alpha-1 antitrypsin. This protein may be isolated in a substantially pure form.

In order to express heterologous proteins in S. pombe, it is desirable to have the expression under control of a promoter which is indigenous to S. pombe. Such a promoter may be the approximately 700 base pair Sph I-Eco RI fragment comprising the S. pombe ADH promoter which may be isolated from plasmid pADHpoI, a plasmid which is disclosed by Russell et al., J.Biol.Chem., 258, 143-149 (1983), the disclosure of which is incorporated by reference herein in its entirety. Other S. pombe promoters may include the glycolytic pathway promoters, such as, triose phosphate isomerase, phosphofructo kinase, glyceraldehyde-3-phosphate dehydrogenase. The heterologous protein selected for expression is alpha-1 antitrypsin, a protease inhibitor present in mammalian blood whose apparently major physiological function is to inhibit elastase, a potent protease which hydrolyzes structural proteins. Alpha-1 antitrypsin may also inhibit other

the blood may be associated with chronic obstructive pulmonary emphysema and infantile liver cirrhosis. Therefore, production of proteins having alpha-1 antitrypsin activity may be useful for treating alpha-1 antitrypsin deficiency and to examine the mechanism of the acute-phase response which occurs under inflammatory conditions in the body whereby the concentration of alpha-1 antitrypsin is substantially increased.

The gene for the predominant form of human alpha-1 antitrypsin is shown in FIG. 1. A DNA fragment comprising such gene which may be utilized may be derived from the plasmid HAT 4. The construction of HAT 4 is illustrated generally in FIG. 3.

To prepare HAT 4, the intermediate vector C1/1 is utilized. Referring to FIG. 3, the S. cerevisiae TPI promoter, described by Alber and Kawasaki, J.Molec.Appl. Genet., 1, 419-434 (1982) is digested with Bal31 exonuclease from the KpnI restriction site within the TPI coding region. The resultant blunt end is converted to a Eco RI sticky end by the addition of a 5' blunt end Eco RI adaptor. A synthetic oligonucleotide linker having the sequence AATTCATGGAGGATCC is ligated to the Eco RI sticky end, then digested with Bam HI. The resultant fragment comprising the TPI promoter may be inserted into the Bam HI site of the vector C1/1. The restriction map of C1/1 is shown in FIG. 4. The C1/1 plasmid was constructed from plasmid pJDB248 (Beggs, Nature, 275, 104-109 (1978)) by removing the pMB9 sequences from pJDB248 by a partial digestion with Eco RI. Eco RI digested pBR322 was then inserted to give C1/1. It will be noted that the linker sequence

... supplies an ATG initiation codon and a GAG

codon, which codes for glutamic acid, the first amino acid of naturally occurring human alpha-1 antitrypsin. Therefore, these codons are "in frame" when the rest of the alpha-1 antitrypsin structural gene is inserted at the Bam HI site.

The gene coding for the predominant form of human alpha-1 antitrypsin (shown in FIG. 1) may be isolated from a human cDNA library using the baboon sequence (Kurachi et al., PNAS USA, 78, 6826-6830 (1981); and Chandra et al., Biochem.Biophys.Res.Com., 103, 751-758 (1981)) as a DNA hybridization probe. This gene may then be inserted as a PstI fragment, as shown in FIG. 3 into vector pUC13 (prepared as described by Vieira et al., Gene, 19, 259-268 (1982) for vectors pUC8 and pUC9, but containing the multiple restriction site shown in FIG. 2 at start of the lacZ gene) at the single PstI site in pUC13. In the resultant construct the antitrypsin gene was followed by an XbaI site and an Eco RI site in the multiple cloning sequence. Therefore, between these two sites was inserted the yeast TPI terminator as a 700 base pair XbaI-Eco RI fragment from pTPIC10. The plasmid pTPIC10 is described by Alber and Kawasaki, J.Molec.App.Gene., 1, 419-434 (1982). The resultant plasmid shown in FIG. 3 is pUC α -1 + FG1 which contains the human alpha-1 antitrypsin with a yeast transcription terminator. A detailed structure of pUC α -1 + FG1 is shown in FIG. 5.

Referring to FIG. 3 an Eco RI, Bam HI synthetic DNA adaptor is then added to the Eco RI site of pUC α -1 + FG1 in order to create a Bam HI site on the 3' end of the

the ... the human ... sequence ... removed by cutting with Bam HI to liberate a fragment of

approximately 2100 base pairs. This Bam HI fragment is then inserted into the modified C1/1 plasmid containing the TPI promoter with Bam HI adaptor. The resultant plasmid HAT 4 contains the TPI promoter, ATGGAG adaptor, human alpha-1 antitrypsin gene and TPI terminator. A detailed structure of HAT 4 is shown in FIG. 6. Referring to FIG. 7, HAT 4 was digested with Eco RI and Hind III and the approximately 1400 base pair Hind III-Eco RI fragment was isolated. This fragment was ligated to the Eco RI-Sph I 700 base pair fragment containing the S. pombe ADH promoter discussed above. The resultant Hind III-Sph I linear segment was then inserted into Sph I and Hind III digested shuttle vector pCAS. Plasmid pCAS is a derivative of CV13 (also known as YEpl3; Broach et al., Gene, 8, 121-123 (1979)) in which the single SstI site has been destroyed by cutting with SstI, blunting the ends with T4 DNA polymerase and dNTP's, and religating the linear molecule. The final construct shown in FIGURE 7 containing the S. pombe ADH promoter, alpha-1 antitrypsin coding sequence, and TPI terminator is known as pR103-5. A detailed structure of pR103-5 is shown in FIG. 8. This plasmid also contains the S. cerevisiae LEU2 gene which complements the leu1-32 mutation of S. pombe.

The plasmid pR103-5 may be used to transform S. pombe strains by conventional techniques. Growth of the transformed cells will result in expression of polypeptides exhibiting the protease inhibition activity of human alpha-1 antitrypsin. These proteins may be useful for treatment of genetic alpha-1 antitrypsin deficiency and other disease states related to inadequate levels of alpha-1 antitrypsin.

Exposure of the lungs of patients with alpha-1 antitrypsin deficiency to alpha-1 antitrypsin may be treated, such as,

chronic obstructive pulmonary disease or adult respiratory distress syndrome. Non-genetically related emphysema may be also be treated, such as, emphysema resulting from heavy smoking. Conditions not necessarily
5 confined to the lungs may also be treated, such as, cystic fibrosis and arthritis. For a review of alpha-1 antitrypsin in deficiency see Gadek, J. E., and R. Crystl, "Alpha-1 Antitrypsin Deficiency", The Metabolic Basis of Inherited Disease, J. B. Stanbury, J. B. Wyngaarden and D. S. Fredrickson, McGraw-Hill, New York,
10 pages 1450-1467 (1982).

The proteins according to the present invention may be admixed with conventional pharmaceutical carriers. Preferably the proteins may be administered intra-
15 venously or by inhalation. While effective dosages may vary according to the severity of the condition and weight of the subject, dosages in the range of 0.5 to 10.0 gm/wk of a protein introduced intravenously may, in many cases, be effective. Lower dosages may be
20 effective if the method of administration is by inhalation. Oral administration may also be effective provided the alpha-1 antitrypsin is protected in capsules or coded carriers from premature degradation in the digestive tract. The following examples set forth
25 specific embodiments according to the present invention, but the invention is not intended to be limited thereto.

EXAMPLE 1

EXPRESSION OF PROTEINS HAVING ALPHA-1 ANTITRYPSIN
ACTIVITY FROM S. POMBE.

... is used to transform S.

No. 20680. The strain is obtained by crossing strain PR131 (h+, leu1-32), described by Kohli et al., Genetics, 87, 471-489 (1977), and strain PRP17 (h-, cdc10-129), described by Nurse et al., Molecular and General Genetics, 146, 167-168 (1976), and isolating clones which give consistent colony size. The transformed cells may be grown under selective conditions described by Russell et al., Nature, 301, 167-169 (1983). RNA analysis using Northern blots (Thomas, PNAS USA, 77, 5201-5205 (1980)) and a ³²P-labeled Eco RI-Hind III alpha-1 antitrypsin-TPI terminator probe indicates that high level of transcription of alpha-1 antitrypsin cDNA is directed by the ADH promoter. Cell extracts from the log phase cultures grown under selective conditions are analyzed by ELISA assay and show 0.85%-0.95% of total soluble protein to be protein exhibiting alpha-1 antitrypsin activity.

EXAMPLE 2

CHARACTERIZATION OF PROTEINS FROM S. POMBE EXHIBITING ALPHA-1 ANTITRYPSIN ACTIVITY.

Alpha-1 antitrypsin proteins from the S. pombe transformants above in Example 1 are further characterized by a purification on amino-adsorption column and electrophoresis on a polyacrylamide/SDS gel. The column is prepared by covalently attaching affinity purified antibodies to human alpha-1 antitrypsin to CNBr-activated Sepharose according to the method of Cuatrecasas, J.Biochem., 245, 3059 (1970). Disrupted cells are extracted with phosphate buffered saline pH 7.2 containing 0.5m NaCl and applied to the column. The

gel in the presence of sodium dodecyl sulfate. The
alpha-1 antitrypsin proteins which are produced in S.
pombe have molecular weights in the range of 42-43 kdal.
Naturally occurring human alpha-1 antitrypsin has a
5 molecular weight of approximately 54 kdal., having a
carbohydrate composition of approximately 16% by weight.
See Hodges et al., J.Biochem., 254, 8208-8212 (1979).
The proteins having alpha-1 antitrypsin activity
produced by S. pombe will thus appear to be
10 unglycosylated or substantially unglycosylated.

EXAMPLE 3

ELISA Assay for AT

To assay for AT, 200 microliters of goat anti-alpha-1-
antitrypsin (10 micrograms/ml in 0.1M Na₂CO₃ pH 9.8)
15 that has been affinity purified is placed in each well
of a 96 well microtiter culture plate at 37°C for two to
four hours. This solution is removed and 200
microliters of phosphate buffered saline (PBS) pH 7.2
containing 1% BSA and .05% Tween 20 is added to each
20 well at 37°C for two hours.

Test samples, including standards, are then added to
each well and AT, if present, binds to the specific
antibody. The wells are rinsed with PBS after 15-30
minutes of incubation. 200 microliters of rabbit
25 anti-alpha-1-AT is added to each well in the presence of
1% BSA in PBS. After 15 minutes incubation, the
solution is removed and the wells are rinsed with PBS.

anti-rabbit IgG
added to each well and incubated for 15 minutes. After
30

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5 rinsing the wells, 200 microliters of a substrate solution was added which contains 30 milligrams of dinitrophenyl phosphate dissolved in 50 ml of 0.1 m diethanolamine pH 9.6. A yellow color is allowed to develop (15-60 minutes) and intensity is recorded at 405 nm on a Titertek Multiscan detector. The yellow color is proportional to the amount of AT in the test solution.

CLAIMS:

1. A method of producing a heterologous polypeptide in yeast Schizosaccharaomyces pombe comprising the step of growing a culture of said yeast transformed by a DNA vector, said vector comprising a segment coding for an S. pombe promotor, wherein expression of said polypeptide is under regulation of said promotor.
2. A method as claimed in claim 1, wherein said promotor comprises the alcohol dehydrogenase promotor.
3. A method as claimed in claim 1 or claim 2, wherein said polypeptide possesses the protease inhibition activity of mammalian alpha-1-antitrypsin.
4. A method as claimed in claim 3, wherein said polypeptide has the amino acid sequence of a form, e.g. the predominant form, of mammalian alpha-1-antitrypsin.
5. A method as claimed in claim 1, wherein said vector comprises plasmid pR103-5.
6. A method as claimed in any one of claims 1 to 5, wherein said polypeptide is substantially unglycosylated.
7. A method as claimed in any one of claims 1 to 6, wherein said polypeptide is substantially pure.
8. A DNA construct comprising a promotor of S. pombe and a gene for a heterologous protein positioned such that expression of said protein in S. pombe is under regulation of said promotor.
9. A construct as claimed in claim 8 and further defined by the specific feature of any one of claims 2 to 4.
10. A DNA construct as claimed in claim 8 comprising the DNA sequence of pR103-5.
11. A method of constructing a DNA vector capable in S. pombe of expressing a heterologous polypeptide, e.g. mammalian alpha-1-antitrypsin, comprising ligating DNA

S. pombe.

12. A transformant strain of S. pombe containing a DNA construct as defined in any one of claims 8 to 10.

13. A method of forming a transformant strain of S. pombe having the capability of producing a heterologous polypeptide, e.g. a polypeptide having the protease inhibition activity of mammalian alpha-1-antitrypsin, which method comprises introducing a DNA transfer vector comprising a construct as defined in any one of claims 8 to 10 into S. pombe to transform the same thereby altering its genotype to encode in expressible format for said polypeptide.

14. A therapeutic formulation for treating disease states related to inadequate amounts of alpha-1-antitrypsin in a mammal comprising a polypeptide which has been produced by a method as claimed in any one of claims 3 to 5 or in claim 6 or claim 7 when dependent directly or indirectly upon any one of claims 3 to 5 formulated for such treatment, e.g. for administration by inhalation, for oral administration or for intravenous administration.

15. A method of producing a therapeutic formulation for treating disease states related to inadequate amounts of alpha-1-antitrypsin in a mammal comprising formulating for such treatment a polypeptide which has been produced by a method as claimed in any one of claims 3 to 5 or in claim 6 or claim 7 when dependent directly or indirectly upon any one of claims 3 to 5.

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5' GGGGGGGGGGGGGG CA CCA CCA CTG ACC
10 20

[illegible]

[illegible]

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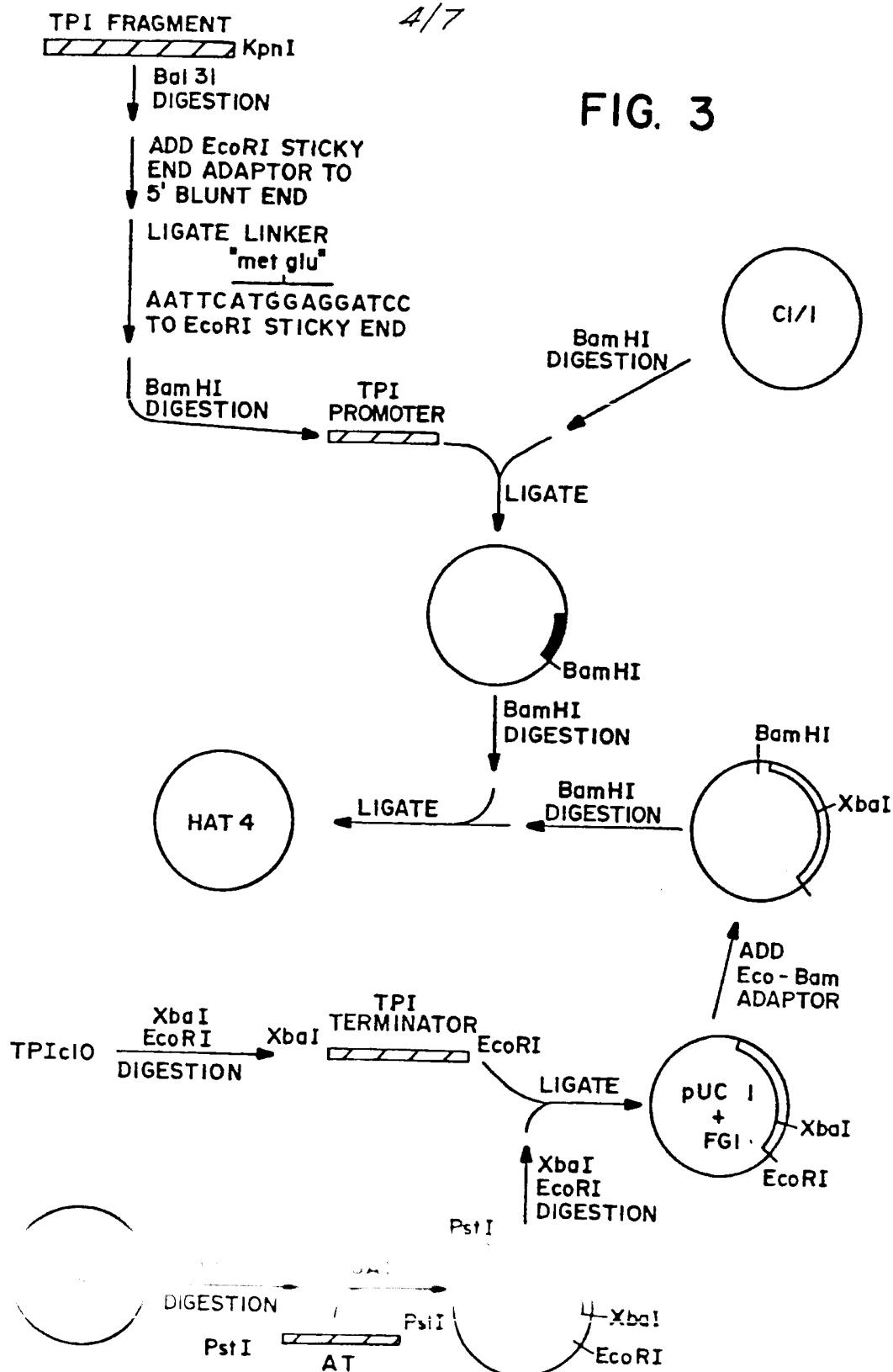
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 THR MET ILE THR
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 AccI, MincII
 ASN SER LEU ALA
 HaeIII
 EcoRI

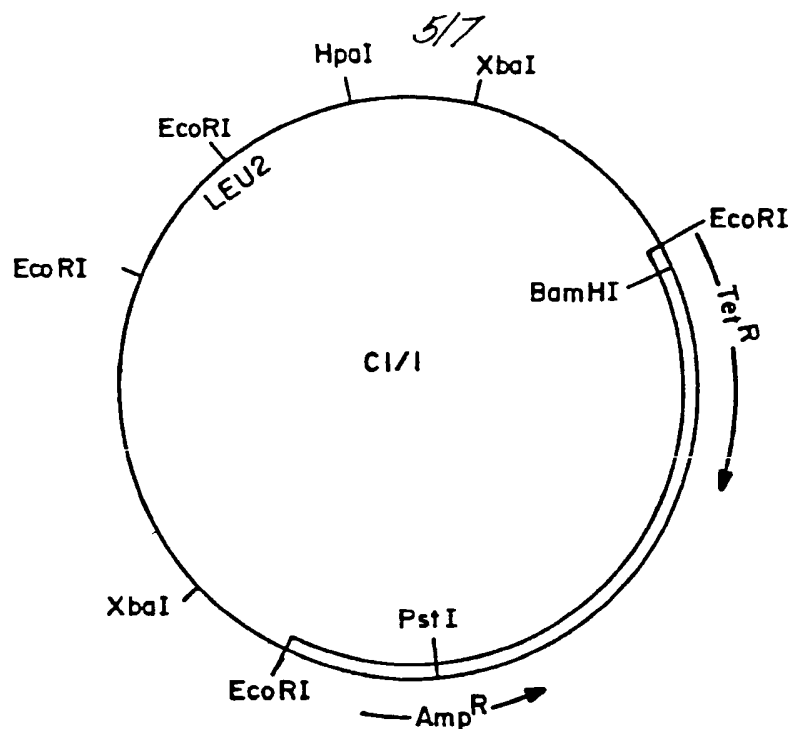
3/7

FIG. 2

M13mp11/pUR

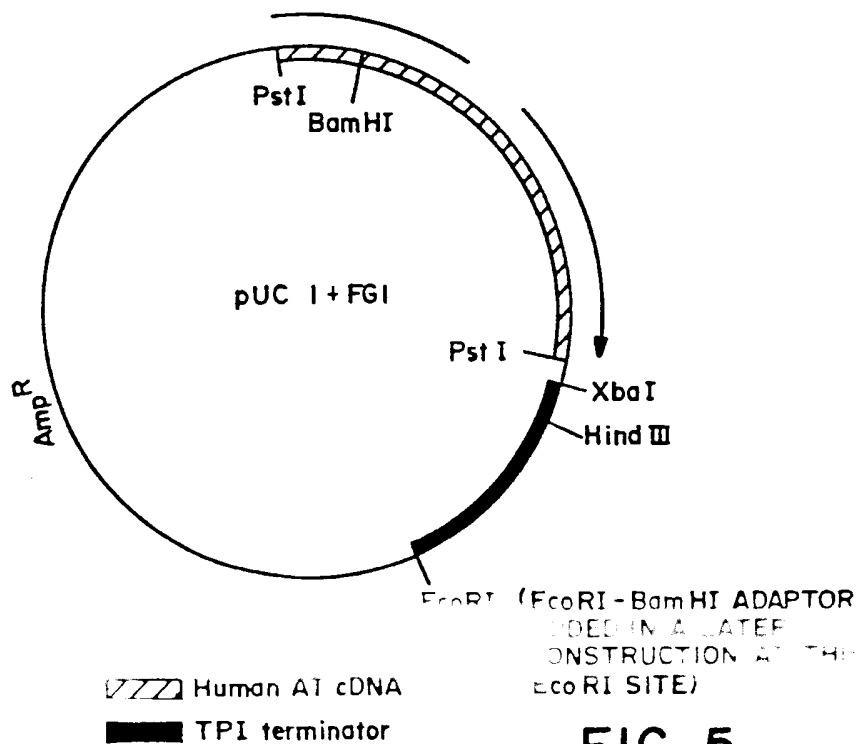
FIG. 3





□ pBR322

FIG. 4



▨ Human AT cDNA

■ TPI terminator

FIG. 5

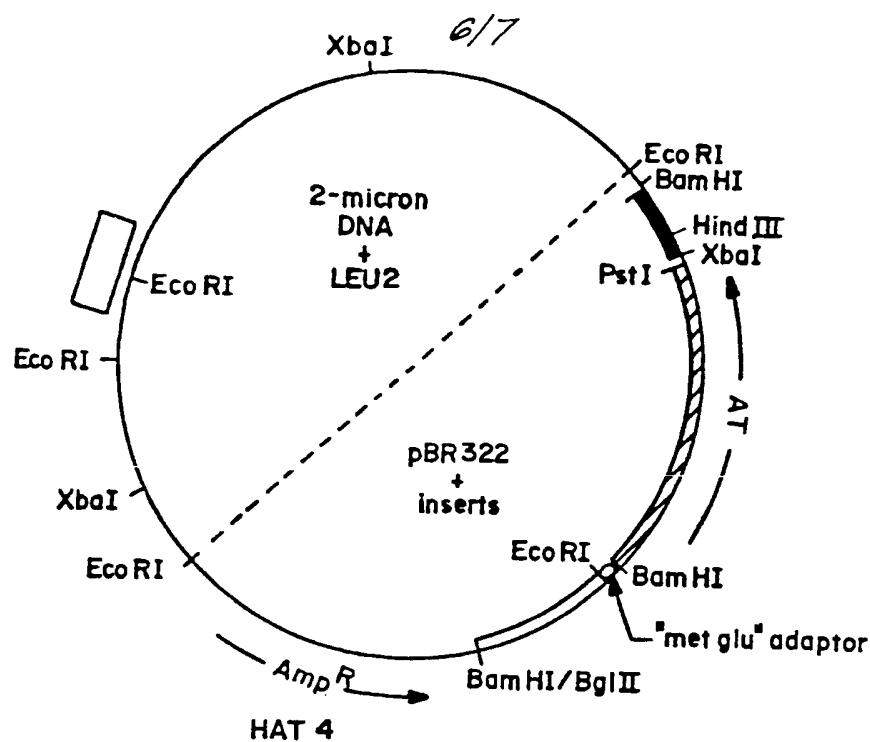


FIG. 6

TPI promoter
 Human AT cDNA
 TPI terminator

PR 103-5

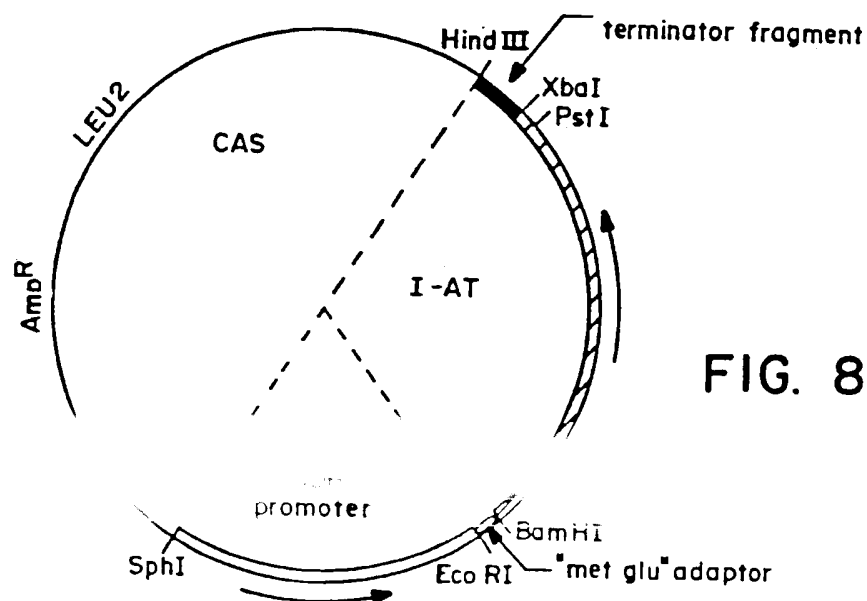


FIG. 8

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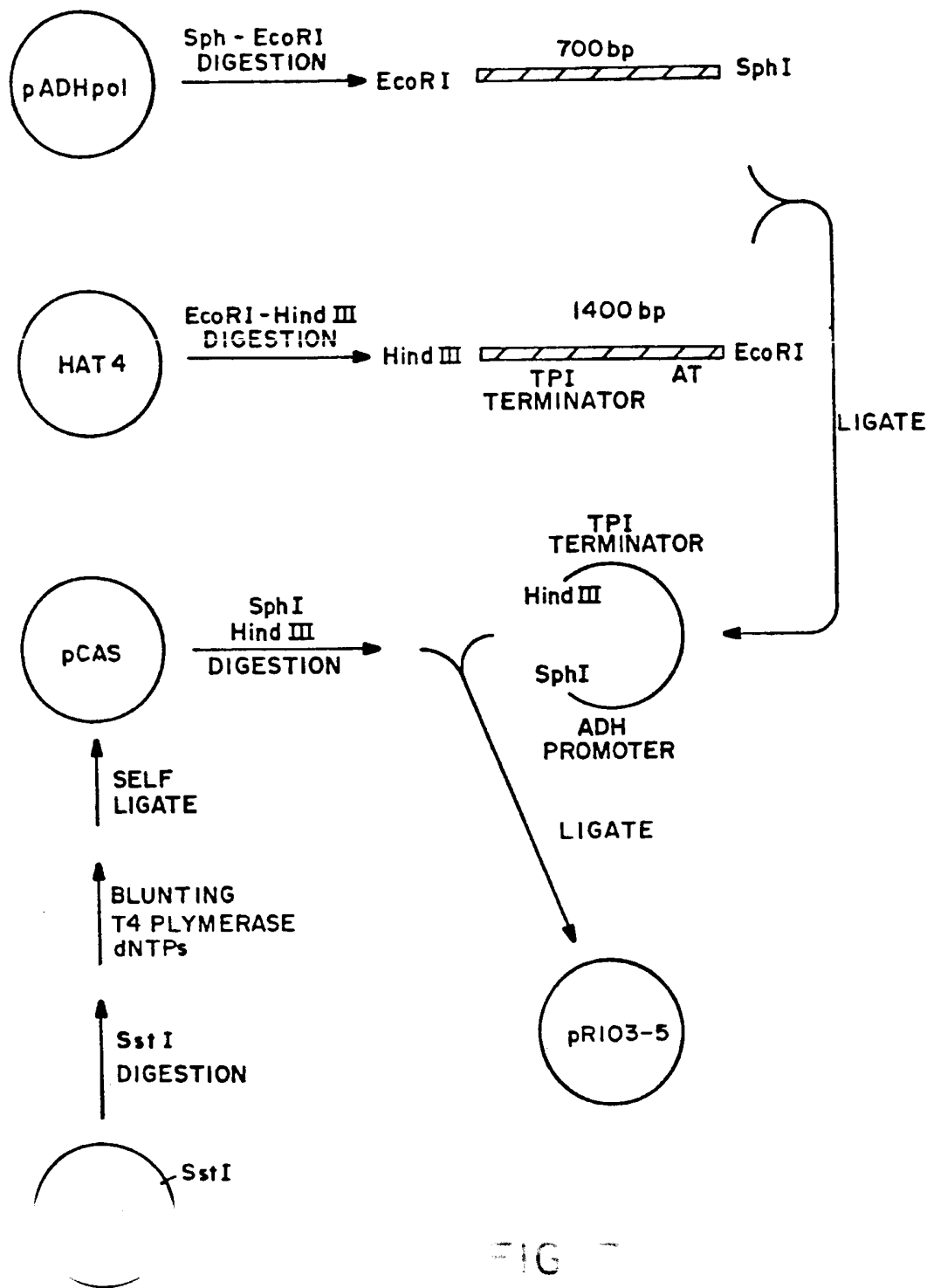


FIG 7



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Application number

DOCUMENTS CONSIDERED TO BE RELEVANT			EP 84305571.6
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	NATURE, vol. 290, no. 5802, March 12, 1981 (New York, London) D. BEACH et al. "High-frequency transformation of the fission yeast Schizosaccharomyces pombe" pages 140-142 * Totality *	1,8, 11,13	C 12 N 15/00 C 12 P 21/00 C 12 N 9/99 A 61 K 37/02 //C 12 R 1/85
A	MOLECULAR & GENERAL GENETICS, vol. 182, no. 3, 1981 (Springer Verlag, Berlin, Heidelberg) M. YAMAMOTO et al. "Cloning of a Gene from the Fission Yeast S. pombe which Complements E. coli pyr B, the Gene for Aspartate Transcarbamylase" pages 426-429 * Totality *	1,8, 11,13	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
D,A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 78, no. 11, November 1981 (Baltimore, USA) K. KURACHI et al. "Cloning and sequence of cDNA coding for α_1 -anti-trypsin" pages 6826-6830 * Abstract *	1,3,4, 11,13	C 12 N C 12 P A 61 K C 07 H
The present search report has been drawn up for all claims			

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CATEGORY OF CITED DOCUMENTS

- X : particularly relevant if taken alone
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- D : document cited in the application
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EUROPEAN SEARCH REPORT

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Application number

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D, A	BIOCHEMICAL AND BIOPHYSICAL RE-SEARCH COMMUNICATIONS, vol. 103, no. 2, November 30, 1981 (New York, London, Toronto, Sydney, San Francisco, Tokyo) T. CHANDRA et al. "Induction of α_1 -Antitrypsin mRNA and cloning of its cDNA" pages 751-758 * Summary * -----	1, 3, 4, 11, 13	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
CATEGORY OF CITED DOCUMENT			
* particularly relevant if taken alone Y particularly relevant if combined with another document of the same category A technological background O non-written disclosure P intermediate document		theory or principle underlying the invention earlier patent document, but published after the filing date D document cited in the application L document cited for other reasons & : member of the same patent family, corresponding document	